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PROTEOLYTIC STUDIES IN LIVER HOMOGENATE IN PRESENCE OF SUBSTITUTED THIOSEMICARBAZONES

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ABSTRACT

A series of substituted benzaldehyde thiosemicarbazones were synthesized and evaluated their effect on endogenous protein hydrolysis in liver homogenate. The results showed that proteolytic enzyme activity depends on the substitution of benzaldehyde and it was observed that *p*-substituted thiosemicarbazones exerted maximum inhibition.

Key words: Thiosemicarbazones, Endogenous proteolysis, Liver homogenate, Proteolysis.

INTRODUCTION

Proteases are vitally important for many physiological processes and therefore have become a main target in drug development. Proteases refer to the various enzymes that digest protein and catalyze the hydrolytic cleavage of peptide bonds. Nature has developed various strategies to protect cells and whole organisms against undesired proteolysis. One of them is the control of proteolytic activity by inhibition[1]. Proteolysis is an irreversible regulatory mechanism and is known to selectively cleave specific proteins. With highly controlled actions, these enzymes play influential roles in DNA replication and transcription, cell proliferation and differentiation, angiogenesis, neurogenesis, ovulation, fertilization, wound repair, stem cell mobilization, hemostasis, blood coagulation, inflammation, immunity, senescence, necrosis and apoptosis[2]. Therefore, desregulation in proteolytic actions underlie many diseases like cancer and neurodegenerative and cardiovascular disorders. Because of proteases' ability to degrade extracellular matrices and proteins, they are strongly associated with cancer progression, specifically invasion and metastasis[3]. With strong evidence of protease involvement in diseases, proteases serve an important role in

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drug development. So there is need for find out the key targeting proteases and their involvement in specific diseases.

Thiosemicarbazones have a wide range of biological activities; therefore these carbonyl derivatives have been the subject of most structural and medical studies. Some of the reported biological activities are antifungal[4], antimalarial[5], antitumoral[6], antiviral[7], antibacterial[8], anticonvulsants[9]and antiprotozoal[10] but there are limited number of studies reported on enzymatic activities. Recently, some studies have been reported related to the effect of these compounds on parasite enzymes. These versatile derivatives have been reported to inhibit *Plasmodium falciparum* and *Trypanosoma sp.*, which are the causative agent of malaria and Chagas's disease[11]. Therefore, increasing prevalence of drug resistant parasites and the growing number of immune compromised individuals are exacerbating the problem to the point that the need for drugs targeting molecules is greater now than ever. So, our present study is focused to find out such compounds which act as active protease inhibitors.

EXPERIMENTAL

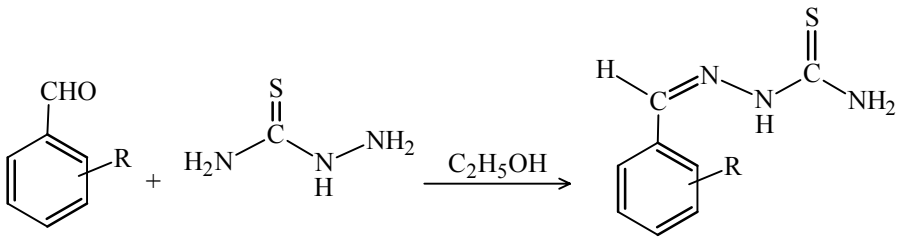
The reactions were monitored by thin layer chromatography. Thin layer chromatography was performed with silica-gel G (suspended in $CHCl_3$-EtOH) and plates were viewed under Iodine vapors. Melting points were determined by electrochemical capillary melting points apparatus and are uncorrected. Elisa plate reader was used for measuring absorbance in the visible range. The spectrofuge was used for centrifugation purpose. IR spectra (KBr, cm^{-1}) were recorded on a Horizon 300 MHz spectrometer. ^1H NMR spectra was recorded on Brucker 300 MHz NMR spectrometer (chemical shifts in δ ppm) using TMS as an internal standard.

General method for the synthesis of thiosemicarbazones

The thiosemicarbazones were synthesized by the usual method[12] followed by Scheme 1. Substituted benzaldehyde (1 mmol) was mixed with thiosemicarbazide (1 mmol) in ethanol. The reaction mixture was heated for 2-4 hours. It was then cooled. The precipitates obtained were filtered, washed with cold water, dried and recrystallized from ethanol. The prepared thiosemicarbazones were analysed by melting point, IR and ^1H NMR (Table 1).

Preparation of acetone powder

The fresh goat liver was treated with chilled acetone in mixer-cum-blender. It was then filtered and dried. Then acetone powder was homogenized in 0.1 M acetate buffer (pH 5.3) containing 1 mM EDTA and 0.2 M NaCl.



R = H, *o*-Cl, *m*- Cl, *p*- Cl, *o*-OCH_3, *m*-OCH_3, *p*-OCH_3, *o*-NO_2, *m*-NO_2, *p*-NO_2

Scheme 1

Table 1: Spectral data of different thiosemicarbazones

	Compound	IR (cm^{-1})	NMR (δ=ppm)
1a	*o*-Chlorobenzaldehyde	N-H stretch (3418, 3248), C=N Strech (1605)	11.61 (1H, s HN), 8.47 (1H, s, CH=N), 8.10 (1H, s, NH/SH), 7.36 (1H, s, NH/SH), 7.38-7.43 (m, 1H,Ar), 7.47-7.50 (1H, m, Ar), 8.28-8.31 (2H, m, Ar)
1b	*m*-Chlorobenzaldehyde	N-H stretch (3379, 3240), C=N Strech (1612)	11.43 (1H, s HN), 8.63 (1H, s, CH=N), 8.01 (1H, s, NH/SH), 8.19 (1H, s, NH/SH), 7.38-7.44 (2H, m, Ar), 7.63-7.65 (2H, m, Ar)
1c	*p*-Chlorobenzaldehyde	N-H stretch (3433, 3279), C=N Strech (1597)	11.47 (1H, s HN), 8.01 (1H, s, CH=N), 8.02 (1H, s, NH/SH), 8.24 (1H, s, NH/SH), 7.44 (2H, d, *J* = 8.4 Hz, Ar), 7.83 (2H, d, *J* = 8.4 Hz, Ar)
1d	*o*-Methoxybenzaldehyde	N-H stretch (3441, 3325), C=N Strech (1597)	11.31 (1H, s HN), 7.99 (1H, s, CH=N), 7.89 (1H, s, NH/SH), 8.09 (1H, s, NH/SH), 7.73 (2H, d, *J* = 8.7 Hz, Ar), 6.96 (2H, d, *J* =8.7 Hz, Ar), 3.88 (3H, s, OCH_3)
1e	*m*-Methoxybenzaldehyde	N-H stretch (3394, 3279), C=N Strech (1589)	11.39 (1H, s HN), 7.43(1H, s, CH=N), 6.96 (1H, s, NH/SH), 8.21 (1H, s, NH/SH), 7.27-7.33 (2H, m, Ar), 8.01-8.05 (2H, m, Ar), 3.79 (3H, s, OCH_3)

Cont…

	Compound	IR (cm^{-1})	NMR (δ=ppm)
1f	*p*-Methoxybenzaldehyde	N-H stretch (3410, 3294), C=N Strech (1589)	11.42 (1H, s HN), 8.40 (1H, s, CH=N), 7.93 (1H, s, NH/SH), 8.13 (1H, s, NH/ SH), 6.93-6.98 (1H, m, Ar), 7.04-7.06 (1H, m, Ar), 7.34-7.39 (1H, m, Ar), 8.07-8.09 (1H, m, Ar), 3.82 (3H, s, OCH_3)
1g	*o*-Nitrobenzaldehyde	N-H stretch (3425, 3240), C=N Strech (1605)	11.71 (1H, s HN), 8.46 (1H, s, CH=N), 8.12 (1H, s, NH/SH), 8.39 (1H, s, NH/SH), 7.60-7.65 (1H, m, Ar), 7.71-7.76 (1H, m, Ar), 8.01-8.03 (1H, m, Ar), 8.42-8.45 (1H, m, Ar)
1h	*m*-Nitrobenzaldehyde	N-H stretch (3394, 3240), C=N Strech (1605)	11.62 (1H, s HN), 8.66 (1H, s, CH=N), 8.19 (1H, s, NH/SH), 8.31 (1H, s, NH/SH), 7.66-7.71 (1H, m, Ar), 8.14-8.16 (1H, m, Ar), 8.23-8.25 (2H, m, Ar)
1i	*p*-Nitrobenzaldehyde	N-H stretch (3487, 3364), C=N Strech (1582)	11.75 (1H, s HN), 8.21 (1H, s, CH=N, 8.08 (1H, s, NH/SH), 8.40 (1H, s, NH/SH), 8.11-8.12 (2H, m, Ar), 8.24-8.26 (2H, m, Ar)
1j	Benzaldehyde	N-H stretch (3418, 3256), C=N Strech (1589)	11.51 (1H, s HN), 8.05 (1H, s, CH=N), 7.98 (1H, s, NH/SH), 8.19 (1H, s, NH/SH), 7.39-7.79 (5H, m, Ar)

Assay for proteolytic activity

The enzyme homogenate was mixed with universal buffer of pH 5.0 and was incubated at 37°C for 24 h. The reaction was stopped by the addition of TCA and the resulting solution was centrifuged to precipitate proteins. The acid soluble proteins were quantitated in the supernatant using biuret reagent[13]. The experiment was conducted in triplicate and the results are presented in the Table 2.

The results shown are % inhibition in presence of 1.0 and 0.1 mM concentrations of compounds. The % inhibition is calculated with respect to the activity in control taken as zero inhibition where no compound was added but an equivalent amount of solvent was present.

Table 2: Effect of different thiosemicarbazones on endogenous proteolytic activity

Thiosemicarbazones	M.P.^{o}C (Lit.)	Effective Conc.	% Inhibition
o-Chlorobenzaldehyde **(1a)**	206-210 (207-210)	1.0	22.07
m-Chlorobenzaldehyde **(1b)**	182-185	1.0	10.38
p-Chlorobenzaldehyde **(1c)**	210-212 (209-212)	1.0	76.62
		0.1	61.03
o-Methoxybenzaldehyde **(1d)**	180-182	1.0	27.27
m-Methoxybenzaldehyde **(1e)**	208-210	1.0	32.46
p-Methoxybenzaldehyde **(1f)**	217-220	1.0	64.93
		0.1	22.07
o-Nitrobenzaldehyde **(1g)**	210-212 (214-215)	1.0	54.54
m-Nitrobenzaldehyde **(1h)**	240	1.0	28.57
p-Nitrobenzaldehyde **(1i)**	240-242	1.0	84.41
		0.1	80.51
Benzaldehyde **(1j)**	160-164 (167-169)	1.0	67.53
		0.1	50.64

RESULTS AND DISCUSSION

The thiosemicarbazoones were synthesized by reacting thiosemicarbazide with substituted benzaldehydes in presence of ethanol by usual method and confirmed by comparing the melting points with literature[11,14,15]. The synthesized products were also confirmed by melting points, IR and ^{1}H NMR listed in Table 1.

In the present study, inhibitory effect of proteases on endogenous protein substrate at pH 5.0 was measured. The liver homogenate was mixed with buffer (pH 5.0) in presence of substituted benzaldehyde thiosemicarbazones at 1.0 mM concentration. After 24 hours of incubation at 37^{o}C, the reaction was stopped by adding TCA solution and the acid soluble proteins were estimated by Biuret reagent.

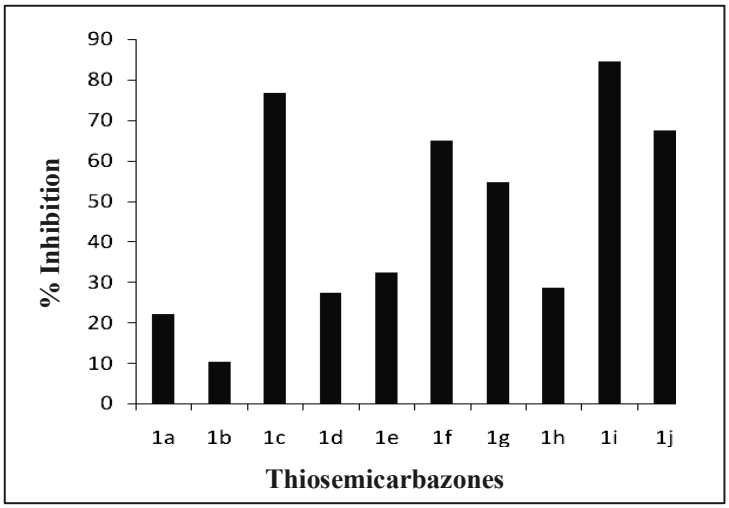


Fig. 1: The results are shown as % inhibition in presence of 1.0 mM of individual thiosemicarbazones (1a-1j). The % inhibition is calculated with respect to the activity in control taken as zero inhibition where no compound was added but an equivalent amount of solvent was present

The endogenous protein hydrolysis study shows that it was maximally inhibited in presence of *p*-substituted thiosemicarbazones as compared with *o*- and *m*-substituted thiosemicarbazones. These results are similar to those observed earlier in our lab as in case of semicarbazones, hydrazones and phenyl hydrazones[16-18]. Among the *p*-substituted thiosemicarbazones, it was found that the effect is more in nitro substituted thiosemicarbazone followed by chloro and methoxy substituted thiosemicarbazone and it follows the order NO_2 > Cl > H > OCH_3. At still lower concentration *i.e.* 0.1 mM concentration, *p*-nitro benzaldehyde thiosemicarbazone inhibited maximumally (80%) followed by *p*-chloro benzaldehyde thiosemicarbazone (61%), benzaldehyde thiosemicarbazone (50%) and *p*-methoxy benzaldehyde thiosemicarbazone showed least inhibition (22%). From results, it was observed that the above proteolytic study is affected by the substitution on the benzaldehyde moiety and the activity was more in the compound containing electron withdrawing group as compared to the compound containing electron releasing group.

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